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Making More of Milk Sugar by Engineering Lactic Acid Bacteria

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ABSTRACT

By exploiting their genetic and metabolic capacity, lactic acid bacteria can be used to generate a variety of products from milk sugar lactose other than the archetypical lactic acid. This review will outline the different genetic and metabolic engineering strategies that can be applied to lactic acid bacteria to realize this valorization of lactose. Attention will be given to the controlled production, by lactic acid bacteria, of enzymes that convert lactose. In addition, we will summarize the progress and potential of redirecting the metabolic flux from lactose to produce existing, modified, and novel metabolites. Examples to be discussed include the production of compounds that are relevant for the flavour, texture, and health aspects of foods. © 1998 Elsevier Science Ltd. All rights reserved

Keywords: lactic acid bacteria; lactose; metabolic engineering; genetic engineering

INTRODUCTION

The first sugar that man and other mammals encounter in their life is O-β-D-galactopyranosyl-(1- > 4)-β-D-glucopyranose, also known as lactose. This disaccharide is a major component of milk but otherwise does rarely occur in nature. Hence, most attention for lactose originates from a dairy perspective and this notably holds for the conversion of this milk sugar by lactic acid bacteria during industrial dairy fermentations. The major end product of these fermentations, by definition, is lactic acid that depending on the used lactic acid bacteria can be produced as a racemic mixture or as one of its stereoisomers. For a long time, D- and L-lactic acid have been considered as the only compounds of interest that could be generated from lactose by lactic acid bacteria. However, in recent years it has been shown that lactose may be converted into a variety of traditional, modified or novel products other than the archetypical lactic acid by exploiting the genetic and metabolic potential of these bacteria. In this review we will provide an overview of the different engineering strategies that are presently used to realize this valorization of lactose, discuss several examples of genetic and metabolic engineering of lactic acid bacteria, and address their use as cell factories in bioreactors, for the *in situ* production in foods, and as delivery systems in the gastro-intestinal tract of the consumer.

In order to appreciate the application potential of the discussed engineering approaches, it is important to note here that lactic acid bacteria have a number of features that make them very suitable candidates for many of the conversions of lactose. These include their long tradition of safe use, their capacity to grow rapidly on lactose-based media derived from milk, and their potential to generate a variety of metabolic products. However, a unique property of lactic acid bacteria relates to their application as starter cultures in industrial food fermentations. This implies that they can convert lactose not only in bioreactors but notably in food products or even the gastro-intestinal tract of consumers of food. Therefore, in the examples discussed below, specific attention will be given to the potential of lactic acid bacteria for the *in situ* production of compounds that contribute to the flavour, texture, or health benefits of foods.

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ENGINEERING APPROACHES

Various strategies may be adopted to exploit lactic acid bacteria for the valorization of lactose. All of these have been advanced by distinct scientific developments that extend beyond the progress made in the past decade on the genetics and biotechnology of this industrially important group of bacteria (Gasson and de Vos, 1994). A major development relevant to all present and future engineering approaches is the analysis of microbial genomes, that in less than three years resulted in the publication of more than 10 complete genome sequences (Doolittle, 1998). Moreover, many other microbial genome sequencing projects are in progress and these also involve the genomes of lactic acid bacteria, such as those belonging to the genera *Lactococcus*, *Lactobacillus* and *Streptococcus*. The sensational stream of sequences

that is emerging allows for the selection of genes coding for existing, modified, and novel conversions of lactose that can be used in the various engineering approaches described here.

One approach is to use lactic acid bacteria for the production of enzymes that catalyse the conversion of lactose into more valuable products with food, pharmaceutical or agricultural applications. This genetic engineering approach has been greatly stimulated by the development of inducible gene expression systems that uncouple growth from the production of enzymes and allows for the efficient expression of heterologous genes (Kok, 1996; Kuipers *et al.*, 1997).

Another avenue is to exploit the physiological potential of lactic acid bacteria to generate products other than lactic acid under specific environmental conditions. This engineering approach has gained significantly from the extensive knowledge on the metabolic pathways of lactic acid bacteria and insights into their physiological control (Hugenholtz 1993, Cocaign-Bousquet *et al.*, 1996; de Vos, 1996). However, since most of these growth or stress conditions are not met during *in situ* production and are even hard to apply in industrial bioreactors, this physiological steering approach is not discussed here.

The combination of genetic and physiological approaches, finally, allows for a wide variety of metabolic strategies by which metabolic fluxes from carbohydrates may be rerouted under conditions that are industrially applicable (de Vos, 1996). Lactic acid bacteria have a relatively simple metabolism and, therefore, are ideal candidates for metabolic engineering, the more so since their basic catabolic pathways are not utilized for synthetic purposes and hence can be modified without direct interference of growth. This may lead to the increased production of traditional compounds, the production of metabolites with improved functionality, or the synthesis of completely novel products. The strategies that have been applied in lactic acid bacteria include single, multiple, and whole pathway engineering, the engineering of redox reactions, and engineering global control systems.

All the genetic and metabolic engineering avenues for making more of the milk sugar lactose will be illustrated below with some examples that have been realized recently, have potential to be achieved soon, or may become a reality in the near future.

PRODUCTION OF LACTOSE-CONVERTING ENZYMES FROM LACTIC ACID BACTERIA

The past decade has seen impressive progress in the development of genetic tools for most lactic acid bacteria, resulting in cloning, expression and in some cases protein secretion systems for *Lactococcus*, *Leuconostoc*, *Streptococcus*, and *Lactobacillus* (de Vos and Simons, 1994; Mercenier *et al.*, 1994; de Vos *et al.*, 1997). Several new developments, however, need to be mentioned here since they have an impact on the genetic engineering of lactic acid bacteria. First, new industrial hosts have been added to the growing list of transformable lactic acid bacteria and these include the yoghurt starter *Lactobacillus bulgaricus*, for which a secretion system has now been reported (Satoh *et al.*, 1997). In addition, a set of defined synthetic promoters covering a wide range of efficiency has been generated for use in lactococci and most likely also other lactic acid bacteria (Jensen and Hammer, 1998). Finally, various new inducible gene expression systems have been created and some have shown to be highly effective for the production of homologous and heterologous enzymes (Kuipers *et al.*, 1997). One of these is of specific interest since it has been shown to be effective in a wide range of lactic acid bacteria. This is the NIsin controlled expression (NICE) system based on the molecular control mechanism by which nisin biosynthesis is autoregulated (Kuipers *et al.*, 1995; de Ruyter *et al.*, 1996a; Kuipers *et al.*, 1998). Two nisin-responsive promoters, preceding the *nisA* and *nisF* genes, have been discovered that may be activated by the addition of food-approved compounds such as the antimicrobial peptide nisin, produced by some strains of *Lactococcus lactis*, on growth media containing nisin, or nisin-containing milk products. When these promoters are coupled to one or more genes of interest, their expression can be modulated in a dynamic range of approximately thousandfold, which allows even lethal genes to be expressed (de Ruyter *et al.*, 1996b, de Ruyter *et al.*, 1997). Signal transduction occurs via a binary communication module consisting of the sensor NisK, which in the presence of the inducer nisin is likely to be autophosphorylated, and the response regulator NisR, a transcriptional activator, that is thought to be only active after phosphorylation by the autophosphorylated NisK. By introducing the *nisRK* genes for this signal transduction pathway in other lactic acid bacteria, effective control of gene expression by the NICE system has been realized in *Lactococcus*, *Leuconostoc*, *Streptococcus*, and *Lactobacillus* (de Ruyter *et al.*, 1996a; Kleerebezem *et al.*, 1997).

To valorize lactose, the described expression and secretion systems for lactic acid bacteria can be used to produce enzymes that convert this disaccharide. Various such enzymes are known and several of these are presently exploited to convert lactose into glucose and galactose, or more complex compounds such as lactulose, lactitol, lactobionic acid, or oligosaccharides (Table 1). Some of these enzymatic conversions, such as the formation of lactitol and lactulose, require the presence of cofactors (Kulbe and Chmiel, 1992). Although the problems associated with cofactor recycling can be solved in various ways, lactic acid bacteria may offer some advantages for *in vivo* conversions since their redox state may be influenced by the oxygen concentration and the level of oxidase production (Lopez de Felipe *et al.*, 1998; see also below). Other catalytic reactions, however, such as those catalysed by glycohydrolases, do not require accessory cofactors. These are particularly relevant for lactic acid bacteria that are well-established producers of β-galactosidases, the key enzymes in lactose degradation (de Vos and Vaughan, 1994). Proof of the concept that lactic acid bacteria can be used to overproduce β-galactosidases was provided 10 years ago with the overexpression of the

Table 1. Lactose valorization by enzymatic conversions

Product	Enzymatic Activity
Hydrolysed Lactose	β-Glycosidase
Lactitol	Aldose reductase
Lactulose	Polyol dehydrogenase
Lactobionic acid	Cellobiose oxidase
Oligosaccharides	β-Glycosidase

Escherichia coli lacZ gene in *Lactococcus lactis* (de Vos and Simons, 1988). Since then, a variety of homologous and heterologous β-galactosidases have been overproduced in lactic acid bacteria (de Vos and Vaughan, 1994). More recently, this has resulted in overproduction of considerable amounts of heterologous glycohydrolases in *L.lactis* (O'Sullivan *et al.*, 1996; de Ruyter *et al.*, 1996a). Noteworthy are attempts to produce glycohydrolases that not only generate glucose and galactose but act as retaining enzymes and have the capacity to synthesize oligosaccharides, which receive a growing interest because of their potential application in functional foods. The production of such oligosaccharides is usually performed at high temperatures that allow high sugar solubility and hence require highly thermostable β-glycosidases. Various of these thermostable enzymes have been found in thermophilic microorganisms and the highest thermal stability and activity is presently found with the glucohydrolase from the hyperthermophilic *Pyrococcus furiosus*, which shows a broad substrate specificity, high β-galactosidase activity, and a potential to synthesize a wide range of products from disaccharides (Voorhorst *et al.*, 1995; Fischer *et al.*, 1996). The NICE system is an ideal system to produce such thermostable enzymes that have the additional advantage of a simple separation from the production hosts by a heat treatment. In addition, also other enzymes with a potential to valorize lactose may be produced using the NICE or other systems. Although *in situ* production of these enzymes on a first glance does not seem to offer many advantages, it should be realized that new markets may ask for an increased degree of lactose hydrolysis in dairy food products. Moreover, lactic acid bacteria that hydrolyse lactose are among the few probiotic bacteria with an established record of efficacy (Marteau and Rambeaud, 1993).

SINGLE-STEP ENGINEERING

One of the simplest forms of metabolic engineering is the inactivation or overexpression of a specific single gene. When a gene for a key metabolic conversion is targeted, this may result in a dramatic change in the metabolic capacity. As a consequence, the metabolic flux may be rerouted resulting in new products to be generated from lactose. Most of the effect is to be expected from a single gene inactivation since this results in the most dramatic change unless there is a redundancy in coding capacity—however, only few examples are known of duplicated genes in lactic acid bacteria (Davidson *et al.*, 1996).

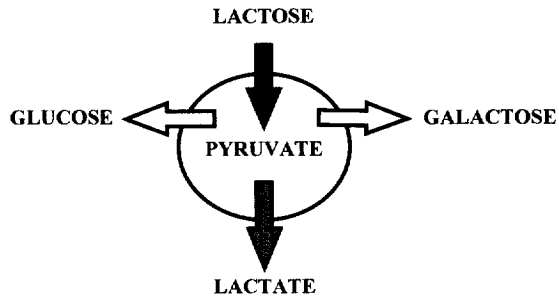

Fig. 1. Simplified representation of essential metabolic conversions of lactose by lactic acid bacteria. Black arrows indicate conversions that generate reducing equivalents (NADH) while the stippled arrows indicate those which consume these equivalents.

The potential of single gene engineering will be illustrated with a simple overview of the metabolic pathways for lactose catabolism in lactic acid bacteria that may be grouped in three major conversions (Fig. 1). The first one is lactose transport and hydrolysis into the constituting glucose and galactose moieties. The second one is formed by a series of reactions from monosaccharides that generate energy, reducing equivalents (NADH), and pyruvate. The last one is the oxidation of pyruvate to lactate by lacate dehydrogenase (LDH) with concomitant utilization of reducing equivalents (NADH) generated in the previous conversion.

A variety of single-step conversions have been engineered in lactic acid bacteria by the use of several techniques, including plasmid curing and random, IS-mediated, and site-directed mutagenesis (Table 2). A variety

Table 2. Engineering of single-step conversions. The following experimental approaches were used to generate these inactivations: P, plasmid curing; M, spontaneous or induced mutagenesis; IS, IS based spontaneous mutation; SDM, site-directed mutagenesis

Lactic Acid Bacteria	Technique	Process	Potential Application	References
Lactococcus lactis	P	Inactivation lactose fermentation	Accelerated cheese ripening	McKay and Baldwin (1990)
	M	Glucokinase inactivation	Glucose accumulation	Thompson *et al.* (1985)
	M	L-LDH Partial inactivation	Increased metabolic potential	McKay and Baldwin (1974)
	SDM	Complete L-LDH inactivation	Increased metabolic potential	Platteeuw *et al.* (1995)
Lactobacillus delbrueckii	IS	Inactivation lactose operon	No post fermentation acidification	Mollet and Hottinger (1990)
	SDM	D-LDH/L-LDH exchange	Only L-lactate production	Sasaki *et al.* (1993)
Streptococcus thermophilus	M	Galactose operon activation	No galactose induced browning	Catzeddu *et al.* (1998)
Lactobacillus helveticus	SDM	Complete D-LDH inactivation	Only L-lacate production	Bhowmik and Steele (1994)
Lactobacillus plantarum	SDM	Complete L-LDH inactivation	Only D-lactate production	Ferrain *et al.* (1994)
	SDM	Simultaneous L- and D-LDH inactivation	Increase metabolic potential	Ferrain *et al.* (1996)

of applications for the thus mutated strains can be envisaged and many of these relate to the *in situ* application of these strains in food products. Only limited regulatory problems may be anticipated with these strains since they can be either produced using self-cloning or do not result in novel foods at all since traditional genetic techniques have been used. Hence, some of the described strains have reached the market place. These include strains which are deficient in lactose utilization due to an impaired lactose transport or hydrolysis that can be used in cheese manufacture to accelerate ripening or in yoghurt production to prevent post-fermentation acidification.

In some cases, there is no direct application of the engineered strains but their metabolic potential may be increased. This applies to the strains of various lactic acid bacteria in which the LDH activity is affected. A strain of *L.lactis* partly defective in LDH activity was characterized following its fortuitous finding 25 years ago (McKay and Baldwin, 1974). This inspired the deliberate construction of LDH-negative strains of *L.lactis* by completely inactivating the single *ldh* gene (Platteeuw *et al.*, 1995). The resulting strains do not produce lactic acid but are viable, have a higher yield, and show a mixed-acid fermentation. Moreover, the metabolic flexibility of the generated *L.lactis* strains is greatly enhanced since the key intermediate pyruvate is no longer channelled into lactic acid (Fig. 1). In fact, high intracellular concentrations of pyruvate accumulate and mainly acetate and ethanol are produced (Platteeuw *et al.*, 1995). Similar phenotypes were found in other lactic acid bacteria, although in some cases two LDH activities, specific for each of the two stereoisomers of lactic acid, had to be eliminated. The ability to construct these LDH-deficient strains has greatly increased the potentiality of metabolic exploitation of lactic acid bacteria as will be illustrated below with the engineering of multiple steps.

MULTIPLE-STEP ENGINEERING

It is evident that engineering of multiple steps offers an infinite set of combinations. Here we will focus on three important products, ethanol, alanine and diacetyl that can be generated from pyruvate in LDH-deficient strains (Fig. 2). In this way, lactose can be converted both in bioreactors and *in situ* in food products into these simple compounds that are known to contribute to flavour, health and conservation.

Engineering the production of ethanol and alanine have the advantage that these products, like lactic acid, are more reduced than pyruvate and hence may act as sinks for reducing equivalents (NADH). In particular, this may be relevant for the *in situ* production in anaerobic food fermentations. Ethanol overproduction was realized by the overexpression of the *Zymomonas mobilis pdc* and *adh* gene cluster encoding the unique pyruvate decarboxylase and an alcohol dehydrogenase from this well-known ethanol producing bacterium. When introduced into LDH-deficient strains, the overexpression of these ethanol-genes resulted in a highly significant production of ethanol in *L.lactis*, amounting up to 2% alcohol (Hugenholtz *et al.*, unpublished results). An extremely high conversion of pyruvate was realized in another engineering approach in which use was made of the *Bacillus sphaericus alaD* gene for an alanine dehydrogenase was overexpressed in combination with the NICE system (Hols *et al.*, 1998). Under appropriate conditions this resulted in the complete transformation of *L.lactis* from a homolactic into a homoalanine fermentation. Moreover, by inactivating the *alr* gene for the alanine racemase, a stereospecific conversion into exclusively L-alanine was realized. This highly successful metabolic engineering exercise illustrates the great potential of relatively simple rerouting involving two or three steps, leading to the production of massive amounts of a completely new compound, alanine, which may both contribute to a sweet flavour and provide a health benefit when applied in foods.

A final example of a multiple step engineering approach is given by the production of the flavour compound diacetyl that is generated from the intermediate α-acetolactate (AL) by spontaneous oxidative decarboxylation (see also Fig. 3). As a consequence, the production of the flavour precursor AL was realized by the overexpression of the *L.lactis als* gene for AL-synthase, which catalyses the condensation of two molecules of pyruvate. When introduced into LDH-deficient strains the overexpression plasmids resulted in a highly significant rerouting of the metabolic flux (approximately 80%) from lactose to the AL-synthesis route (Platteeuw *et al.*, 1995). In a later study, the utility of the *ilvBN* gene, coding for another AL-synthetase with higher affinity for pyruvate than the *als* gene product, was evaluated and its expression resulted in a similar but less pronounced rerouting of pyruvate to AL (Benson *et al.*, 1996). However, a considerable limitation in these approaches formed the fact that the generated AL was further converted into acetoin and butanediol, a more reduced product

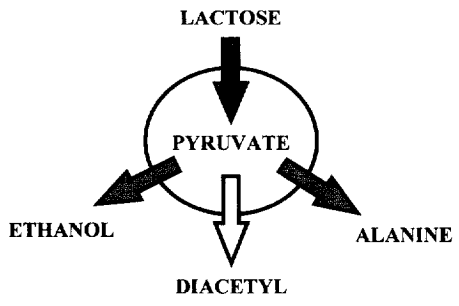

Fig. 2. Simplified representation of metabolic engineering of pyruvate metabolism in lactic acid bacteria. Labelling of arrows as in Fig. 1.

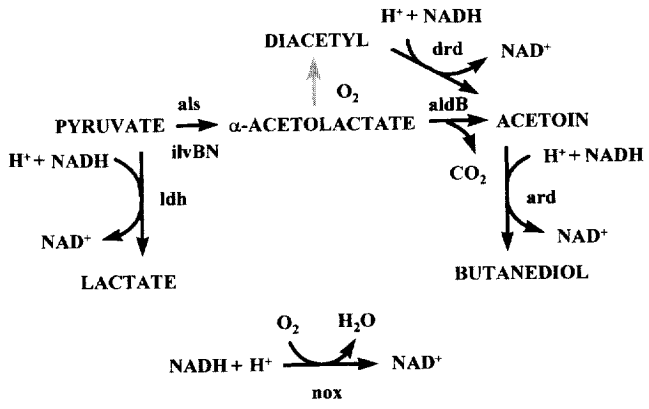

Fig. 3. Schematic representation of the conversions relevant for diacetyl engineering and their genetic nomenclature.

(Fig. 3). Simple *in papyrus* analysis would predict that this could be prevented by the inactivation of the *aldB* gene for the AL decarboxylase (Goupil-Feullerat *et al.*, 1997). Although such inactivations could be realized in wild-type strains, it appeared impossible to combine an *aldB* inactivation with an *ldh* inactivation in rapidly growing cells. This example illustrates the advantages and also the limitations of metabolic engineering. However, as described below, alternative approaches could be implemented to realize the final aim of rerouting lactose to the production of diacetyl or its precursor AL.

WHOLE PATHWAY ENGINEERING

A great challenge for now and the future is provided by whole pathway engineering and this has been recently initiated with studies to engineer the production of polysaccharides that have potential as biothickner or immunostimulant in fermented foods or as neutraceuticals. The production of exopolysaccharide (EPS) has received considerable attention since it is naturally produced from lactose by the so called 'ropy' lactic acid bacteria. The complexity of its biosynthesis can be illustrated by the analysis of the organization and control of the 12-kb *epsRABCDEFGHIJKL* gene cluster of *L. lactis* involving 14 coordinately transcribed genes coding for an unique phosphorylated polysaccharide (van Kranenburg *et al.*, 1997) (Fig. 4). The biosynthesis of the backbone of the repeating unit of this EPS has recently been elucidated and the function of the glycosyl transferase EpsD, EpsE and EpsF, and EpsG has been identified (van Kranenburg *et al.*, 1997 and 1998). By introducing plasmids carrying this *eps* operon into *L. lactis* strains, the capacity to produce the specific EPS has been transferred. Presently, these plasmids are combined with inactivations of important genes such as *galE*, which is involved in the interconversion of glucose and galactose

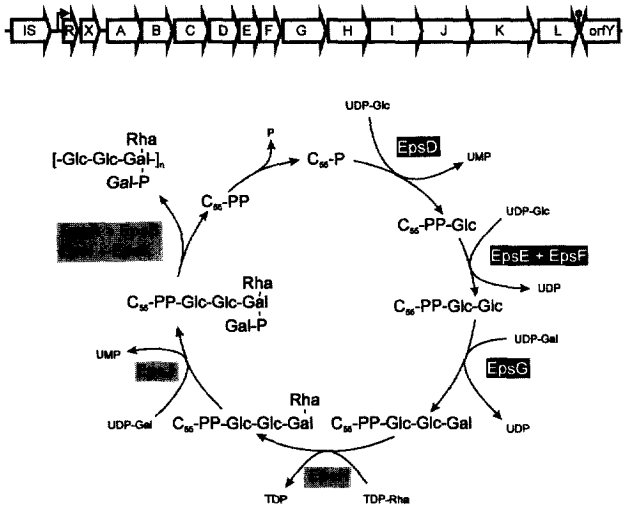

Fig. 4. Genetic organization of the *eps* gene cluster (top) and a model for EPS biosynthesis (bottom) for *L.lactis* NIZO B40. The functionality of the black-boxed enzymes in the indicated conversions is based on experimental evidence, while that of the grey-boxed enzymes is based on homology predictions as well as on the clustering and coordinate expression of their genes. C55-P, lipid carrier; Glc, glucose; Gal, galactose.

and hence may affect the composition of the EPS (Grossiord *et al.*, 1998). Moreover, heterologous and homologous complementation of the *epsD* gene, coding for the first step in the biosynthetic pathway, has been realized allowing for a further engineering of EPS production. Similar EPS gene clusters are found in other lactic acid bacteria and that of *S. thermophilus* has been expressed in *L. lactis* resulting in low but significant EPS production (Stingele *et al.*, 1996). Finally, a recent report describes a high level of polysaccharide production in *L. lactis* upon expression of the *S. pneumoniae* genes for a capsular polysaccharide (Wells *et al.*, 1998). Various other approaches to engineering EPS production may be envisaged and this is expected to develop into an important research objective since *in situ* polysaccharide production is relevant for product properties such as texture and mouthfeel, but also may affect GI-tract passage, colonization, and other potential probiotic properties, as well as stimulate the immune system and function as adjuvant in oral immunizations.

REDOX ENGINEERING

The fermentative sugar metabolism of lactic acid bacteria implies that under anaerobic conditions all reduction equivalents formed during sugar oxidation have to be transferred to the generated end products. Hence, it is expected that any change in the redox balance may affect the metabolic flux. Therefore, we have designed *L.lactis* strains where we can control the redox balance. This was realized by exploiting the NICE system since effective control of gene expression in a dynamic range appeared to be important. The *Streptococcus mutans nox* gene for an effective water-forming NADH-oxidase (NOX) was overexpressed in the NICE system and in combination with specific aeration and induction conditions this allowed for the control of the cofactor balance NADH/NAD (Lopez de Felipe *et al.*, 1998). Application of the metabolic control theory had already predicted that engineering the NADH/NAD ratio had potential for favouring diacetyl production (Fig. 3) (Hugenholtz and Snoep, unpublished data). Deliberate variations in the NOX activity, which could be boosted by the addition of the cofactor FAD, provoked a shift from homolactic to mixed-acid fermentation during aerobic sugar metabolism (Lopez de Felipe *et al.*, 1998). At highest induction levels, pyruvate flux was redistributed from lactic acid to significant amounts of acetoin and diacetyl. Fortunately, the NICE system overexpressing the *nox* gene could be introduced in strains defective in *aldB* (see above). This resulted in highly efficient aroma-producing *L.lactis* strains in which about half of the pyruvate was converted into the desired aroma compound or its precursor AL (Hols *et al.*, unpublished results).

Many key conversions in the central metabolism of lactic acid bacteria are redox reactions involving NADH as a cofactor. The predominant role of the NADH/NAD ratio in the fermentation pattern of a non-dairy strain of *L.lactis* was recently illustrated (Garrigues *et al.*, 1997). As a consequence, it is likely that redox engineering, as shown here with the NICE system, will have an important impact on future engineering studies aimed at understanding the metabolic fluxes and their control as well as generating new products from lactose.

Table 3. Future applications of metabolic engineering of LAB

Product	Potential dairy application
Compatible Solutes	Stress tolerant starters
Oligosaccharides	Probiotic or vaccine strains
Vitamins	Health products
Antioxidants	Health products
Biosurfactants	Low fat products

GLOBAL CONTROL ENGINEERING

The insight in, the number, and the complexity of global control systems that may affect many metabolic conversions in microbial cells in increasing steadily. An important control system is termed catabolite repression since its concerns the preferential catabolism of some readily metabolizable compound such as glucose. Recent insight in catabolite repression in Gram-positive bacteria has led to the identification of a major regulatory protein, termed catabolite control protein, CcpA, encoded by the *ccpA* gene (Deutscher *et al.*, 1995; Kuester *et al.*, 1996). We have now characterized the *ccpA* gene of several lactic acid bacteria, including *L.lactis*, and found that it shows both activating or repressing activities in this host (Luesink *et al.*, 1998). Remarkably, we found that the important *las* operon which includes the three glycolytic genes, *pyk-pfk-ldh*, is activated by CcpA. By inactivating the *L.lactis ccpA* gene, strains have now been generated which have reduced expression of the *las* operon and hence show a mixed acid fermentation, illustrating the role of catabolite repression and global control (Luesink *et al.*, submitted for publication).

FUTURE PROSPECTS

This overview of the current approaches and results on the genetic and metabolic engineering of lactic acid bacteria illustrates that the flexibility of lactic acid bacteria is much larger than expected and that a wide variety of simple and complex compounds can be generated from lactose. Time has come now to move beyond the first excitement of successful metabolic engineering and address the question as to what new concepts could be developed or what further useful products could be made. A few examples that illustrate the potential for future applied research are given here (Table 3). To come from pathway shaking to product making, it is essential that we continue to develop new concepts, implement predictive models for metabolic control, and integrate the genetic, physiological and biochemical expertise with appropriate support from process engineers and product developers.

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REFERENCES

Benson, K. K., Godon, J. J., Renault, P., Griffin, H. G. and Gasson, M. J. (1996) Effect of *ilvBN*-encoded α-acetolactate synthase expression on diacetyl production in *Lactococcus lactis*. *Applied Microbiology and Biotechnology* **45,** 107–111.

Bhowmik, T. and Steele, J. L. (1994) Cloning, characterization and insertional inactivation of the *Lactobacillus helveticus* D(-)lactate dehydrogenase gene. *Applied Microbiology and Biotechnology* **41,** 432–439.

Catzeddu P, Vaughan EE, Deiana P and de Vos WM (1998) Transcriptional regulation and mutations that activate expression of the galactose operon in *Streptococcus thermophilus*. Submitted for publication.

Cocaign-Bousquet, M., Garrigues C., Loubiere, P. and Lindley, N. D. (1996) Physiology of pyruvate metabolism in *Lactococcus lactis*. *Antonie van Leeuwenhoek* **70,** 253–267.

Davidson, B. E., Kordias, N., Dobos, M. and Hillier, A. J. (1996) Genomic organization of lactic acid bacteria. *Antonie van Leeuwenhoek* **70,** 161–183.

Deutscher, J., Kuster, E., Bergstedt, U., Charrier, V. and Hillen, W. (1995) Protein-kinase dependent HPr/CcpA interaction links glycolytic activity to carbon catabolite repression in Gram-positive bacteria. *Molecular Microbiology* **15,** 1049–1053.

De Ruyter, P. G. G. A., Kuipers, O. P., Beerthuyzen, M. M., van Alen-Boerrigter, I. J. and de Vos, W. M. (1996)a Controlled gene expression systems for *Lactococcus lactis* with the food-grade inducer nisin. *Applied and Environmental Microbiology* **62,** 2662–2667

De Ruyter, P. G. G. A., Kuipers, O. P., Beerthuyzen, M. M., van Alen-Boerrigter, I. J. and de Vos, W. M. (1996b) Functional analysis of promoters in the nisin gene cluster of *Lactococcus lactis*. *Journal of Bacteriology* **178,** 3434–3439.

De Ruyter, P. G. G. A., Kuipers, O. P., Meijer, W. M. and de Vos, W. M. b (1997) Food-grade controlled lysis of *Lactococcus lactis* for accelarated cheese ripening. *Nature Biotechnology* **15,** 976–979.

De Vos, W. M. and Simons, G. (1988) Molecular cloning of lactose genes in dairy lactic streptococci: the phospho-β-galactosidase and β-galactosidase genes and their expression products. *Biochimie* **70,** 461–473.

De Vos, W. M. and Vaughan, E. E. (1994) Genetics of lactose metabolism in lactic acid bacteria. *FEMS Microbiological Reviews* **15,** 217–239.

De Vos, W. M. and Simons, G. (1994) Gene cloning and expression systems in lactococci. In: *Genetics and Biotechnology of Lactic Acid Bacteria*, eds M. J. Gasson and W. M. de Vos, Chapman and Hall, London, UK, pp. 52–105.

De Vos, W. M. (1996) Metabolic engineering of sugar catabolism in lactic acid bacteria. *Antonie van Leeuwenhoek* **70,** 223–242.

De Vos, W. M., Kleerebezem, M. and Kuipers, O. P. (1997) Expression systems for industrial Gram-positive bacteria with low guanine and cytosin conten. *Current Opinion in Biotechnology* **8,** 547–553.

Doolittle, R. F. (1998) Microbial genomes opened up. *Nature* **392,** 339–342.

Fahey, R. C., Brown, W. C., Adams, W. B. and Worsham, M. B. (1978) Occurerence of glutathione in bacteria. *Journal of Bactiology*, **133,** 1126–1129.

Ferain, T., Garmyn, D., Bernard, N., Hols, P. and Delcour, J. (1994) *Lactobacillus plantarum ldhL* gene: overexpression and deletion. *Journal of Bacteriology* **176,** 596–601.

Ferain, T., Schanck, A. N. and Delcour, J. (1996) Nuclear magnetic resonance analysis of glucose and citrate end products in an *ldhL-ldhD* double knowck-out strain of *Lactobacillis plantarum*. *Journal of Bacteriology* **178,** 7311–7315.

Fischer, L., Bromann, R., Kengen, S. W. M., de Vos, W. M. and Wagner, F. (1996) Catalytic potency of β-glucosidase from *Pyrococcus furiosus* in glucoconjugate synthesis. *Bio/Technology* **14,** 888–91.

Garrigues, C., Loubiere, P., Lindley, N. D. and Cocaign-Bousquest, M. (1997) Control of the shift from homolactic acid to mixed-acid fermentation in *Lactococcus lactis*: predominant role of the NADH/NAD ratio. *Journal of Bacteriology* **179,** 5282–5287.

Gasson, M. J. and de Vos, W. M. (1994) *Genetics and Biotechnology of Lactic Acid Bacteria.* Chapmann and Hall, Glasgow, UK.

Goupil, N., Corthier, G., Ehrlich, S. D. and Renault, P. (1996) Imbalance in leucine flux and its use for the isolation of diacetyl-overproducing strains. *Applied and Environmental Microbiology* **62,** 2636–2640.

Goupil-Feuillerat, N., Cocaign-Bousquet, M., Godon, J.-J., Ehrlich, S. D. and Renault, P. (1997) Dual role of α-acetolactate decarboxylase in *Lactococcus lactis* subp. *lactis. Journal of Bacteriology* **179,** 6285–6293.

Grossiord, B., Vaughan, E. E., Luesink, E. J. and de Vos, W. M. (1998) Genetics of galactose utilization via the Leloir pathway in lactic acid bacteria. *Le Lait* **78,** 77–84.

Hols, P., Kleerebezem, M., Hugenholtz, J., Ferain, T., Delcour, J. and de Vos, W. M. (1998) Conversion of *Lactococcus lactis* homolactic into homoalanine fermentation through metabolic engineering. Submitted for publication.

Hugenholtz, J. (1993) Citrate metabolism in lactic acid bacteria. *FEMS Microbiological Reviews* **12,** 165–178.

Jensen, P. R. and Hammer, K. (1998) The sequence of spacers between consensus sequences modulates the strength of prokaryotic promoters. *Applied and Environmental Microbiology* **64,** 82–87.

Kleerebezem, M., Beerthuyzen, M. M., Vaughan, E. E., de Vos, W. M. and Kuipers, O. P. (1997) Controlled gene expression systems for lactic acid bacteria: transferable nisin-inducible expression cassettes for *Lactococcus, Leuconostoc* and *Lactobacillus* spp. *Applied and Environmental Microbiology* **63,** 4581–4584.

Kok, J. (1996) Inducible gene expression and environmentally regulated genes in lactic acid bacteria. *Antonie van Leeuwenhoek* **70,** 129–145.

Kuipers, O. P., Beerthuyzen, M. M., de Ruyter, P. G. G. A., Luesink, E. J. and de Vos, W. M. (1995) Autoregulation of nisin biosynthesis in *Lactococcus lactis* by signal transduction. *Journal of Biological Chemistry* **270,** 27229–27304.

Kuipers, O. P., de Ruyter, P. G. G. A., Kleerebezem, M. and de Vos, W. M. (1997) Controlled overproduction of proteins by lactic acid bacteria. *Trends in Biotechnology* **15,** 135–140.

Kuipers, O. P., de Ruyter, P. G. G. A., Kleerebezem, M. and de Vos, W. M. (1998) Quorum sensing controlled gene expression in lactic acid bacteria. *Journal of Biotechnology*, in press.

Kulbe, K. D. and Chmiel, H. (1992) Coenzyme-dependent carbohydrate conversions with industrial potential. *Annals of the New York Academy of Sciences* **542,** 444–464.

Kuester, E., Luesink, E. J., de Vos, W. M. and Hillen, W. (1996) Immunological cross-reactivity to catabolite control protein CcpA from *B. megaterium* is found in many Gram-positive bacteria. *FEMS Microbiol Letters* **139,** 109–115.

Luesink, E. J., Kuipers, O. P. and de Vos, W. M. (1998) Regulation of the carbohydrate metabolism in *Lactococcus lactis* and other lactic acid bacteria. *Le Lait* **78,** 69–76.

Lopez de Felipe, F., Kleerebezem, J., de Vos, W. M. and Hugenholtz, J. (1998) Co-factor engineering in *Lactococcus lactis. Journal of Bacteriology*, in press.

Marteau, P. and Rambaud, J.-C. (1993) Potential of using lactic acid bacteria for therapy and immune modulation in man. *FEMS Microbiological Reviews* **12,** 207–221.

McKay, L. L. and Baldwin, K. A. (1974) Altered metabolism of *Streptococcus lactis* C2 deficient in lactate dehydrogenase. *Journal of Dairy Science* **57,** 181–186.

McKay, L. L. and Baldwin, K. A. (1990) Applications for biotechnology: present and future improvements in lactic acid bacteria. *FEMS Microbiological Reviews* **87,** 3–14.

Mercenier, A., Pouwels, P. and Chassy, B. M. (1994) Genetic engineering of lactobacilli, leuconostocs and *Streptococcus thermophilus.* In: M. J. Gasson, and W. M. de Vos. *Genetics and Biotechnology of Lactic Acid Bacteria,* eds Chapmann & Hall, Glasgow, pp. 252–295.

Mollet, B. and Hottinger, H. (1992) Yoghurt contenant de microorganismes vivants. European Patent Application 0 518 096.

O'Sullivan, D., Walker, S. A., West, S. G. and Klaenhammer, T. R. (1996) Development of an expression strategy using to trigger explosive plasmid amplification and gene expresion. *Bio/Technology* **14,** 82–87.

Platteeuw, C., Hugenholtz, J., Starrenburg, M., van Alen-Boerrigter, I. J. and de Vos, W. M. (1995) Metabolic engineering of *Lactococcus lactis*: influence of the overproduction of α-acetolactate synthase in strains deficient in lactate dehydrogenase as a fuction of culture conditions. *Applied and Environmental Microbiology* **61,** 3967–3971.

Sasaki Y, Ito Y and Sasaki T (1993) Gene conversion in transconjugants of *Lactobacillus delbruecki* subsp. *bulgaricus* using pAMβ1 as an integration vector. *FEMS Microbiological Reviews* **12,** P9.

Satoh, E., Ito, Y., Sasaki, Y. and Sasaki, T. (1997) Application of the extracellular α-amylase gene from *Streptococcus bovis* 148 to construction of a secretion vector for yoghurt starter strains. *Applied and Environmental Microbiology* **63,** 4593–4596.

Stingele, F., Neeser, J.-R. and Mollet, B. (1996) Identification and characterization of the *eps* (exopolysaccharide) gene cluster from *Streptococcus thermophilus* Sfi6. *Journal of Bacteriology* **178,** 1680–1690.

Thompson. J., Chassy, B. M. and Egan, W. (1985) Lactose metabolism in *Streptococcus lactis*: studies with a mutant lacking glucokinase and mannose-phosphotransferase activities. *Journal of Bacteriology* **162,** 217–223.

Van Kranenburg R., Marugg, J. D., van Swam, I. I., Willem, N. J. and de Vos, W. M. (1997) Molecular characterization of the plasmid-encoded *eps* gene cluster essential for exopolysaccharide biosynthesis in *Lactococcus lactis.* Molecular Microbiology **24,** 387–397.

Van Kranenburg R., van Swam, I. I., Marugg, J. D., Kleerebezem, M. and de Vos, W. M. (1998) Exopolysaccharide biosynthesis in *Lactococcus lactis* NIZO B40: functional analysis of the glycosyltransferase genes involved in synthesis of the polysaccharide backbone. Submitted for publication.

Voorhorst, W. G. B., Eggen, R. I. L., Luesink, E. J. and de Vos, W.M. (1995) Characterization of the *celB* gene coding for β-glucosidase from the hyperthermophilic archaeon *Pyrococcus furiosus* and its expression and site-directed mutagenesis. *Journal of Bacteriology* **177,** 7105–7111.

Gilbert, C., Robinson, K., Le Page R. W. L. and Wells, J. M. (1998) Heterologous biosynthesis of pneumococcal type 3 capsule in *Lactococcus lactis* and immunogenecity studies. *Proceedings 5th ASM on the Genetics and molecular biology of streptococcis, enterococci and lactococci,* p. 67.